Plant taxa visited by the European orchard bee *Osmia cornuta* (Latreille, 1805) analyzed by metabarcoding through nesting residuals (Hymenoptera, Anthophila, Megachilidae)

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Abstract. – Wild pollinator populations are in global decline. Two main factors influence the presence of wild pollinators in a given environment: the availability of nesting resources and the availability of floral resources. However, information on the dietary niches of these insects is lacking, and the protocols set up in this context are often costly and lethal. By studying the species *Osmia cornuta* (Latreille, 1805), we aimed to find out whether other methods of sampling the flowers visited by this model species were possible —as they were already tested on other Megachilidae species in previous literature. Using metabarcoding, we sought to better define the floral diversity of our model's diet and how to optimize this definition by combining DNA markers. To do this, we used the inter-cocoon residues of *O. cornuta* from three nesting tubes of private insect hotels located in the same geographical area. Despite the small number of samples, the results of the present study are consistent with previous articles on this species, which used microscopy or metabarcoding with a single marker: the *O. cornuta* diet was here composed of 37 taxa including 12 different families. Although further, larger-scale analyses are needed to support these initial descriptive results, it seems that the topology of the floral communities visited by the mason bee *O. cornuta* can be easily determined by metabarcoding analyses of the residues found in the nesting cavities.

Résumé. – **Les taxons de plantes visités par l'osmie cornue *Osmia cornuta* (Latreille, 1805) analysés par métabarcoding à l'aide de résidus de nidification (Hymenoptera, Anthophila, Megachilidae)**. Les populations de pollinisateurs sauvages connaissent un déclin global. Deux facteurs principaux influencent la présence des pollinisateurs sauvages dans un environnement donné : la disponibilité en ressources de nidification et la disponibilité en ressources florales. Mais les informations sur les niches alimentaires de ces insectes sont lacunaires, et les protocoles mis en place dans ce cadre souvent coûteux et létaux. À travers l'étude de l'espèce *Osmia cornuta* (Latreille, 1805), nous avons cherché à savoir si d'autres méthodes d'échantillonnage des fleurs visitées par cette espèce modèle étaient possibles — ayant déjà été testées sur d'autres espèces de Megachilidae dans la littérature. Ainsi, grâce à l'utilisation du métabarcoding, nous avons cherché à mieux définir les plantes constituant le régime alimentaire de notre modèle et comment optimiser cette définition en cumulant les amorces ADN. Nous avons pour cela utilisé les résidus inter-cocons de trois tubes de nidification d'*O. cornuta* provenant d'hôtels à insectes de particuliers sur une même zone géographique. Malgré un faible

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nombre d'échantillons, les résultats de la présente étude sont cohérents avec les précédents articles sur cette espèce qui utilisaient la microscopie ou le métabarcoding avec une seule amorce : le régime alimentaire d'*O. cornuta* était ici composé de 37 taxons appartenant à 12 familles différentes. Bien que d'autres analyses à plus grande échelle soient nécessaires pour étayer ces premiers résultats descriptifs, il semble que la topologie des communautés florales visitées par l'abeille *O. cornuta* puisse être aisément déterminée par des analyses de métabarcoding sur les résidus trouvés dans les cavités de nidifications.

Keywords. – Mason bee, insect hotel, floral resource, DNA marker.

The honeybee *Apis mellifera* (Linnaeus, 1758) is probably the easiest bee species to study. The practical advantages of studying the honeybee, in particular its ease of management and its ability to visit a very wide variety of flowers (Crane, 1990), have largely contributed to making it a preferred study model in the scientific literature (e.g. Bell *et al.*, 2016 ; Cochard *et al.*, 2021). However, this focus on this species has created taxonomic biases that are likely to undermine interest in the other species, which have been much less studied (Wood *et al.*, 2020): the lack of detailed information on the habitats, diets and occurrence of the other species considerably complicates their conservation (Nieto *et al.*, 2014 ; Schatz *et al.*, 2021). Indeed, there are 983 species of wild bees in France (Ropars *et al.*, 2025), 2,138 in Europe (Ghisbain *et al.*, 2023), and more than 20,000 worldwide (Winfree *et al.*, 2011). Moreover, the study model that is *A. mellifera* has some notable characteristics that set it apart from other bee species. Most other bee species have very different ecologies: they do not live in colonies, have a smaller flight radius, have a shorter flight period and have a more restricted diet (Falk & Lewington, 2015). It is, therefore, necessary to find a new model organism with characteristics closer to those of the majority of wild bees (e.g. solitary, flying not far from the nest, having short flight periods) and with robust populations for experiments and ethics (Wood *et al.*, 2020).

European orchard bees *Osmia cornuta* (Latreille, 1805) are bees of the Megachilidae family (Hymenoptera: Antophila: Megachilidae), known as mason bees. Males measure between 9 and 12 mm, while females measure between 12 and 15 mm (Bellman, 2019). This cavity-nesting species lives particularly in pasture landscapes with little exposure to the wind (Krunić & Stanisavljević, 2006). European orchard bees are polylectic bees (Amiet *et al.*, 2004) meaning that they collect pollen from many different flower species (Cane & Sipes, 2006). The flight period extends from March to July in their natural distribution area (Amiet *et al.*, 2004). Some species of mason bees can fly up to 600 m around their nests (Zurbuchen *et al.*, 2010), but it has recently been shown that the average flight radius of *O. cornuta* is 100 m (Hofmann *et al.*, 2020).

As a wild bee species, *O. cornuta* can suffer from the presence of managed pollinators: from the presence of managed honeybees, it has been shown that mason bees visit fewer flowers and that their food niche diminishes (Hudewenz & Klein, 2015), as for several other wild bee species (Mallinger *et al.*, 2017). However, the ease with which these Megachilidae can be managed using insect hotels has led to an increase in their populations, with a five-fold increase in their abundance in less than 10 years in some European countries (Krunić & Stanisavljević, 2006). *Osmia cornuta* can therefore sometimes be considered a managed species (Bosch *et al.*, 2021).

While we can manage *O. cornuta* populations, this makes it an ideal model organism for the development of new techniques, with a view to applying them to "completely wild" bees in the future. Although Gezon *et al.* (2015) have shown that sampling methods for wild pollinators, even lethal ones, do not significantly alter the structure

of bee communities, it is still wise to test any recent method whose effectiveness is not well known on model species *O. cornuta*, in order to minimize any potential impact on wild bee populations.

But an alternative technique makes it possible to determine the food niche of pollinators and its variability. While this determination has largely been carried out using the sweep net method in the literature (Gay *et al.*, 2024) —a method that is costly in terms of time and resources— metabarcoding offers an effective alternative for analysing the diet of pollinating insects, even if it also remains perfectible (Bell *et al.*, 2023). By examining the plant DNA present in pollen, honey or other matrices, this technique enables identifying a wide range of flower species visited and obtaining accurate results (Hawkins *et al.*, 2015 ; Prosser & Hebert, 2017). Metabarcoding has been used extensively in *A. mellifera* honey and pollen (Bell *et al.*, 2016, 2023), but only rarely in wild bees pollen, and, if it has been done, it has not always been to determine their diet (Keller *et al.*, 2013 ; Rothman *et al.*, 2020). However, some studies exist on certain mason bee species from Megachilidae family, and using several DNA molecular markers to get more accurate results (Richardson *et al.*, 2019): for example Crone *et al.* (2023) on *O. cornifrons* (Radoszkowski, 1887) (a North American species), Crone *et al.* (2025) on *Heriades truncorum* (Linnaeus, 1758) (a species that is very common in the continental climate of Central Europe) and Fernandes *et al.* (2022) on several species of Australian *Megachile* Latreille, 1802. Studying the Megachilidae species one by one is essential to characterize this family foraging behaviour, as it is known that it includes species with very distinct feeding behaviours (from specialist to highly generalist species, Haider *et al.* (2014), Crone *et al.* (2025)). But only few literature explored the diet of the present model species *O. cornuta* through metabarcoding, such as Casanelles-Abella *et al.* (2022) that found 33 species of plants in its diet and Kratschmer *et al.* (2020) that found 16 species, but both using only one DNA molecular marker. This literature on *O. cornuta* diet highlighted that metabarcoding enabled to find a high proportion of plants from Sapindaceae, Rosaceae, Salicaceae, Aceraceae, Fagaceae and Ranunculaceae, especially *Sorbus*, *Salix*, *Acer* and *Quercus* genera (Kratschmer *et al.*, 2020 ; Casanelles-Abella *et al.*, 2022). Another study on *O. cornuta* diet —but using microscopy and not metabarcoding— highlighted *Prunus*, *Salix* and *Acer* as major plants in its diet, and little amounts of *Juglans*, *Quercus* and *Sorbus* genera (Eckerter *et al.*, 2022). Moreover, metabarcoding on *O. cornuta* in previous literature was invasive for individuals or could disrupt their life cycles (e.g. opening of cocoons during diapause (Kratschmer *et al.*, 2020), mimicry of spring temperatures in laboratory).

In this study, we tried to non-lethally determine the plant composition of the diet of *O. cornuta* and the diversity of its plant interaction partners through samples of nesting residues from the same geographical area in France, and a control sample from a distant country and a neighbouring species (*O. bicornis* (Linnaeus, 1758)). We aimed to identify the flowers visited using the environmental DNA method and three different molecular markers —rather than one in the previous literature on *O. cornuta*— and compared the composition of the plants detected. We hypothesized a more common plant composition between French samples than with the control sample, and expected to find a similar composition in terms of families or genera as the previous studies on this bee species. Although the molecular markers were as universal as possible, we also expected slight variations in plant composition between markers, as each marker has amplification affinities with certain sequences. Using the shortest markers used in terms of number of base pairs, we also expected greater

detection of taxa than with longer markers. We therefore sought to begin completing previous literature on other Megachilidae species, and to better guide future research on non-lethal metabarcoding on *O. cornuta*.

Materials and methods

Sampling units. – The French insect hotels' tubes containing the *O. cornuta* in diapause and the inter-cocoon residues —destined to be collected— came from the metropolitan network of a private company, which kindly provided the study material. This company from Charente-Maritime (South-West of France) sells insect hotels to private individuals all over mainland France, and then, in order to maintain their fill rate year after year, recovers the cocoons in diapause and re-dispatches them to each region concerned for the following year. The tubes supplied for the present study were therefore of anonymous origin, out of respect for the company's customers. However, as the company works in separate regions for the storage and re-export of cocoons, we knew that the tubes supplied came from the same geographical area in France (probably South-West). The study was designed to use 10 filled tubes, as supplied by the company, but a very high rate of parasitism coupled to some tubes without enough nesting residuals forced us to select only three.

An additional sample, called control sample, was sent from a geographical area that we had chosen as being very distant and very different in terms of flora: a private company providing insect hotels in Helsinki (Finland) kindly sent a tube hosting a neighbouring species, *Osmia bicornis* —as *O. cornuta* does not nest in Nordic countries.

The tubes were 9 mm in diameter, suitable for nesting by *O. cornuta* and *O. bicornis*. In each tube, one female has deposited pollen and nectar for her eggs, forming a loose, dry whole (Krunić & Stanisavljević, 2006) .

Sampling process. – We extracted the pollen and faecal residues from the mason bees in the laboratory, under a light-flow hood to limit particulate contamination of the samples (fig. 1a). The three cardboard tubes were cut lengthways using a pair of scissors and a scalpel (Sedivy *et al.*, 2011). The tubes were filled to varying degrees, with some cells where the larva had failed to hatch and which enabled us to obtain the required quantity of residues to analyse. Once the tubes had been carefully opened, the remaining cocoons were removed and set aside for sampling (fig. 1b). We sampled both faeces and pollen as in Fernandes *et al.* (2022) but we preferred pollen residues to faeces when the number of residues was sufficient. The pollen pellets left between the cocoons corresponded to remains not ingested by larvae before their diapause in the cocoon or to remains not ingested by non-viable larvae (Filipiak & Filipiak, 2020). The diapausing cocoons were then redeposited in an empty insect hotel and in blank tubes outdoors. Their hatching few weeks later enabled us to determine that they belong to *O. cornuta* species (for French samples) or *O. bicornis* species (for Finnish sample).

Metabarcoding analysis. – The samples were frozen in liquid nitrogen and a mechanical disruption of the cells was performed with ceramic beads on a RETSCH Mixer Mill 200. A second cell disruption was performed with SDS-based buffer and heat treatment. After protein precipitation with potassium acetate, the DNA was precipitated in isopropanol. We performed PCR amplification for *trn*L c–h (i), *trn*L g–h (ii) and ITS2 (iii), respectively with the following primers : (i) trnL c–h-F: CGAAATCGGTAGACGCTACG (Taberlet *et al.*, 1991), *trn*L c–h-R: CCATTGAGTCTCTGCACCTATC (Taberlet *et al.*, 2007), (ii) *trn*L g–h-F: GGGCAATCCTGAGCCAA, *trn*L g–h-R: CCATTGAGTCTCTGCACCTATC (Taberlet *et al.*, 2007 for both R and F), (iii) ITS2-F: GACTCTCGGCAACGGATATC

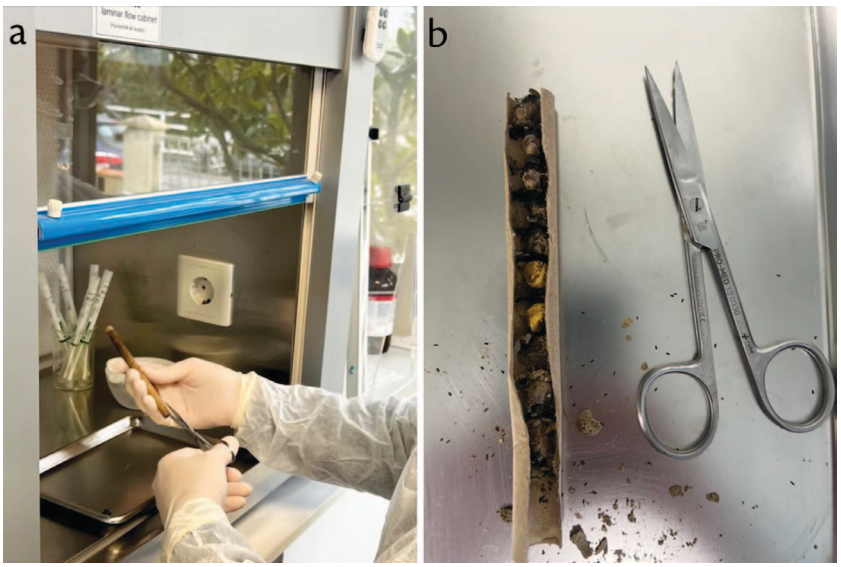

Fig. 1. – Photographs of the protocol for cutting insect hotel tubes. – **a**, Under a light flow hood. – **b**, After complete longitudinal opening of one of the tube.

(Hanssen *et al.*, 2011), ITS2-R: TCCTCCGCTTATTGATATGC (White *et al.*, 1990). The library preparation was performed with Illumina Nextera XT Index Kit v2. After quality control (fragment analyser and qPCR) the library is sequenced on MiSeq Illumina platform.

The raw data were analysed with internal scripts based on *FROGS v.4.1 pipeline* (Escudié *et al.*, 2018 ; Bernard *et al.*, 2021). It consisted of starting with merging the pair-end reads and demultiplexing the primers by trimming them. The Operational Taxomic Units (OTUs) (Sneath & Sokal, 1973) clustering was obtained from *swarm software* (Mahé *et al.*, 2014) with a defined distance of one, which induced the production of OTUs close to Amplicon Sequencing Variants (ASVs) (Eren *et al.*, 2013). Filters were applied to eliminate the chimera (Haas *et al.*, 2011). We then run the methodology of Richardson *et al.* (2019, 2020) redesigned for plant families: we excluded families detected at less than 0.001 proportional abundance (0.1%) in each sample, as it is well known that arbitrary number of reads used as thresholds (e.g. < 10 reads) are less efficient than proportions (Drake *et al.*, 2022). A last filter was applied to the data: removing families identified with only one of the three markers for each sample —as recommended by Richardson *et al.* (2019, 2020). The taxonomic assignment was performed with the blastn algorithm from the *BLAST software v.2.13.0+* (Altschul *et al.*, 1990), querying the NCBI Nt database (updated in November 2023).

Only taxa going to the family, the genus or the species level were kept in the data: all less precise taxa were removed.

A lack of data for the samples metabarcoded with the marker ITS2 is visible in the results section. Environmental DNA is often degraded, therefore fragmented and preventing the amplification of long markers. This is probably why ITS2, the longest marker that was used in the present study (400 bp long), could have difficulties amplifying markers. Furthermore, we did not observe any inhibition of the PCR reaction as all the samples worked on at least one of the markers. The absence of amplification on this marker was multifactorial: it was probably explained more by the genetic material source than by a non-functional reaction.

Analyses. – Even with filters, DNA analysis of vascular plants is generally prone to errors, particularly in taxonomic classification (Quaresma *et al.*, 2024), a known problem with metabarcoding (Cuff *et al.*, 2022 ; Drake *et al.*, 2022). To check the plant identifications obtained by DNA and highlight possible sources of confusion if the data were not examined in detail, we used a compilation of the distribution maps from naturalist web platforms that are *gbif.org*, *plantnet.org* and *inaturalist.org* to verify the accuracy of our results —an important step often neglected.

We then focused on the diet of *O. cornuta* in the different sites by characterizing the proportion of flower families (in terms of number of reads of DNA) for each marker separately and plotting an upset graph.

To better represent the difference in the detection potential of flower species between the three markers, we calculated the number of detected taxa and then the number of these identifications up to the species for each marker and each site.

A correspondence analysis (CA) was conducted using the R package *ca* (Nenadic & Greenacre, 2007). This method was applied to a contingency table comparing the categorical variables of DNA marker identity and the flower family detected. The tabular data were visualized graphically using a biplot in the form of a point cloud on two perpendicular coordinate axes (Greenacre, 2007).

Analyses were run with *R software v.4.3.2* (R Core Team, 2024).

Results

Verification of the accuracy of plant detections with public distribution maps. – The composition of foraged plants by bee individuals highlighted some characteristic plants that enabled to differentiate the French samples and the Finnish control sample (table I): for instance, the presence of species such as *Washingtonia robusta* H.Wendl., 1883, or *Quercus ilex* L., 1753, was characteristic from a South country such as France, whereas *Caragana frutex* (L.) K.Koch, 1869, or *Viscaria alpina* (L.) G.Don, 1831, were some plants that mostly took place in the North of Europe, especially in Finland. As the samples came from private gardens in France or from a urban environment in Finland, we found a sizeable proportion of non-native plants, but these were perfectly plausible as they are often sold in garden centers or online: *Quercus castaneifolia* C.A.Mey., 1831 (an Iranian ornamental species), *Paeonia obovata* Maxim., 1859 (a Japanese ornamental species), *Acer cissifolium* Siebold & Zuccarini, 1865 (a Japanese ornamental species), *Pinus taeda* L., 1753 (a cultivated species from North America), *Couroupita guianensis* Aubl., 1775 (a South American ornamental species), etc. We also found misidentified species, such as *Radermachera frondosa* Chun & F.C.How, 1958, in France, more likely to belong to the species *Radermachera sinica* (Hance) (Hemsl., 1902) —which is widely sold as ornamental plant in garden centers— or *Juglans hopeiensis* Hu, 1932, in Finland which could better be *Juglans mandshurica* Maxim., 1856, known as present in Finnish cities. Few identifications appeared to be false, but curiously they have been repeated between markers (i.e. detected by several markers), which led to believe that they are errors in the international DNA databases rather than a failure of the sequences assignment process: this was the case in particular for *Delavaya toxocarpa* Franch., 1886 (an endemic species from South-East Asia) and for the Chrysobalanaceae family (an exclusively tropical family).

Overview of the diversity of* Osmia cornuta *foraged plant families versus control condition. – The *O. cornuta* individuals have visited different flowers, but the composition and diversity of the individuals diet between markers was quite

Table I. – Floral composition of *Osmia cornuta* (Latreille) diet (individuals 1, 2, 3) and control *O. bicornis* diet (control individual) and the respective taxonomic resolution for each taxa, according to the DNA marker used (ITS2, *trn*L g–h or *trn*L c–h) and the number of DNA reads. DNA data filters: identifications that were found in at least two markers out of three for the same sample, and identifications from the family whose number of reads exceed 0.1% for a given sample and for a given marker.

Individual	Molecular marker	Last taxon	Family	Species	Number of reads
Individual 1	ITS	Species	Fagaceae	*Quercus castaneifolia*	444
Individual 1	ITS	Species	Fagaceae	*Quercus cerris*	1092
Individual 1	ITS	Species	Fagaceae	*Quercus ilex*	234
Individual 1	ITS	Species	Pinaceae	*Pinus nigra*	117
Individual 1	ITS	Species	Sapindaceae	*Acer pseudoplatanus*	40079
Individual 1	ITS	Genus	Fagaceae	*Quercus* sp.	2949
Individual 1	ITS	Genus	Sapindaceae	*Aesculus* sp.	1648
Individual 1	trnL c-h	Species	Fagaceae	*Quercus cerris*	73608
Individual 1	trnL c-h	Species	Sapindaceae	*Delavaya toxocarpa*	683
Individual 1	trnL c-h	Genus	Fagaceae	*Fagus* sp.	114
Individual 1	trnL c-h	Genus	Rosaceae	*Geum* sp.	16
Individual 1	trnL c-h	Genus	Sapindaceae	*Acer* sp.	129161
Individual 1	trnL c-h	Family	Fagaceae	-	2552
Individual 1	trnL c-h	Family	Magnoliaceae	-	2987
Individual 1	trnL c-h	Family	Rosaceae	-	1611
Individual 1	trnL c-h	Family	Sapindaceae	-	7002
Individual 1	trnL g-h	Species	Fagaceae	*Castanopsis carlesii*	20
Individual 1	trnL g-h	Species	Fagaceae	*Quercus robur*	23
Individual 1	trnL g-h	Species	Sapindaceae	*Acer cissifolium*	662
Individual 1	trnL g-h	Species	Sapindaceae	*Acer pictum*	140
Individual 1	trnL g-h	Species	Sapindaceae	*Delavaya toxocarpa*	289
Individual 1	trnL g-h	Genus	Fagaceae	*Fagus* sp.	430
Individual 1	trnL g-h	Genus	Fagaceae	*Quercus* sp.	136953
Individual 1	trnL g-h	Genus	Magnoliaceae	*Magnolia* sp.	10071
Individual 1	trnL g-h	Genus	Rosaceae	*Prunus* sp.	183939
Individual 1	trnL g-h	Genus	Rosaceae	*Rosa* sp.	7
Individual 1	trnL g-h	Genus	Sapindaceae	*Acer* sp.	292604
Individual 1	trnL g-h	Family	Magnoliaceae	-	137
Individual 1	trnL g-h	Family	Rosaceae	-	12460
Individual 1	trnL g-h	Family	Sapindaceae	-	11877
Individual 2	trnL c-h	Species	Arecaceae	*Washingtonia robusta*	1
Individual 2	trnL c-h	Species	Bignoniaceae	*Radermachera frondosa*	1
Individual 2	trnL c-h	Family	Lythraceae	-	3
Individual 2	trnL c-h	Family	Arecaceae	-	2
Individual 2	trnL c-h	Family	Chrysobalanaceae	-	1
Individual 2	trnL g-h	Species	Arecaceae	*Washingtonia robusta*	2
Individual 2	trnL g-h	Family	Arecaceae	-	2
Individual 2	trnL g-h	Family	Bignoniaceae	-	1
Individual 2	trnL g-h	Family	Chrysobalanaceae	-	3
Individual 2	trnL g-h	Family	Lythraceae	-	16
Individual 3	trnL c-h	Species	Cupressaceae	*Chamaecyparis lawsoniana*	58
Individual 3	trnL c-h	Species	Lecythidaceae	*Couroupita guianensis*	1
Individual 3	trnL c-h	Family	Pinaceae	-	29
Individual 3	trnL c-h	Family	Cupressaceae	-	19
Individual 3	trnL c-h	Family	Poaceae	-	6
Individual 3	trnL g-h	Species	Cupressaceae	*Chamaecyparis lawsoniana*	106
Individual 3	trnL g-h	Family	Lecythidaceae	-	1
Individual 3	trnL g-h	Family	Poaceae	-	2

Table I. – *(Continued).*

Individual	Molecular marker	Last taxon	Family	Species	Number of reads
Individual 3	trnL g-h	Family	Cupressaceae	-	21
Individual 3	trnL g-h	Family	Pinaceae	-	32
Control individual	ITS	Species	Caryophyllaceae	*Viscaria alpina*	291
Control individual	ITS	Species	Caryophyllaceae	*Viscaria atropurpurea*	26
Control individual	ITS	Species	Fabaceae	*Caragana frutex*	26
Control individual	ITS	Species	Fabaceae	*Trifolium repens*	1
Control individual	ITS	Species	Fagaceae	*Quercus aliena*	30
Control individual	ITS	Species	Fagaceae	*Quercus hartwissiana*	6
Control individual	ITS	Species	Fagaceae	*Quercus robur*	10
Control individual	ITS	Species	Juglandaceae	*Juglans hopeiensis*	21
Control individual	ITS	Species	Paeoniaceae	*Paeonia obovata*	13
Control individual	ITS	Species	Paeoniaceae	*Paeonia veitchii*	498
Control individual	ITS	Species	Papaveraceae	*Chelidonium majus*	27
Control individual	ITS	Species	Pinaceae	*Picea glauca*	35
Control individual	ITS	Species	Pinaceae	*Pinus contorta*	7
Control individual	ITS	Species	Pinaceae	*Pinus hwangshanensis*	6
Control individual	ITS	Species	Pinaceae	*Pinus massoniana*	10
Control individual	ITS	Species	Pinaceae	*Pinus nigra*	121
Control individual	ITS	Species	Pinaceae	*Pinus taeda*	2
Control individual	ITS	Species	Rosaceae	*Rosa canina*	54
Control individual	ITS	Species	Rosaceae	*Sorbus aucuparia*	9
Control individual	ITS	Genus	Juglandaceae	*Juglans* sp.	1923
Control individual	ITS	Genus	Pinaceae	*Pinus* sp.	1601
Control individual	ITS	Genus	Rosaceae	*Rosa* sp.	62
Control individual	trnL c-h	Species	Fagaceae	*Castanea sativa*	1486
Control individual	trnL c-h	Genus	Fagaceae	*Quercus* sp.	35580
Control individual	trnL c-h	Genus	Juglandaceae	*Juglans* sp.	213
Control individual	trnL c-h	Genus	Pinaceae	*Pinus* sp.	1260
Control individual	trnL c-h	Genus	Pinaceae	*Pseudotsuga*	1
Control individual	trnL c-h	Family	Juglandaceae	-	8
Control individual	trnL g-h	Species	Fagaceae	*Quercus dentata*	12
Control individual	trnL g-h	Species	Rosaceae	*Cercocarpus rzedowskii*	3
Control individual	trnL g-h	Species	Rosaceae	*Crataegus monogyna*	203
Control individual	trnL g-h	Genus	Fagaceae	*Castanea* sp.	5761
Control individual	trnL g-h	Genus	Rosaceae	*Malus* sp.	24
Control individual	trnL g-h	Genus	Rosaceae	*Prunus* sp.	14
Control individual	trnL g-h	Genus	Rosaceae	*Pyrus* sp.	1
Control individual	trnL g-h	Genus	Rosaceae	*Spiraea* sp.	4
Control individual	trnL g-h	Family	Fagaceae	-	73010
Control individual	trnL g-h	Family	Juglandaceae	-	422

similar (fig. 2). The individual 1 had a high proportion of Sapindaceae in its floral spectrum regardless of the marker (47.0% for *trn*L g–h; 62.9% for *trn*L c–h; 89.6% for ITS2), followed by Fagaceae (21.2% for *trn*L g–h; 35.0% for *trn*L c–h; 10.1% for ITS2) and *trn*L g–h highlighted a third of Rosaceae that was not detected by other markers. The individual 2 had nearly a half of its diet composed of Lythraceae for both *trn*L c–h and *trn*L g–h (respectively 37.5% and 66.7%), and the rest of its floral spectrum was composed of Areacaceae (37.5% for *trn*L c–h; 16.7% for *trn*L g–h) and Chrysobalanaceae (12.5% for *trn*L c–h; 12.5% for *trn*L g–h) as well as Bignoniaceae (12.5% for *trn*L c–h; 4.2% for *trn*L g–h). The individual 3 foraged nearly three quarters

of Cupressaceae (68.1% for *trn*L c–h; 78.4% for *trn*L g–h) and a non-negligible proportion of Pinaceae (25.7% for *trn*L c–h; 19.8% for *trn*L g–h) with residuals of Poaceae (5.3% for *trn*L c–h; 1.2% for *trn*L g–h) and Lecythidaceae (0.9% for *trn*L c–h; 0.6% for *trn*L g–h). Surprisingly, results were not consistent between the three markers for the control sample, whereas they were for all the French samples: the ITS2 plants detection gave a very different floral composition from *trn*L markers. The only families detected in common between the ITS2 marker and the *trn*L markers were detected in incomparable proportions: whereas with the *trn*L markers almost all the diet of the control individual was composed of Fagaceae (96.2% for *trn*L c–h; 99.2% for *trn*L g–h), this family represented only 1.0% with ITS2; whereas with ITS2 we found 40.7% of Juglandaceae, this represented only 0.3% with *trn*L g–h. In addition, in this Finnish control individual from the species *O. bicornis*, we found the presence of families much visited by individuals of *O. cornuta* in France: like individual 1, a high proportion of Fagaceae composed its diet, and also a third of Pinaceae (37.3% for *trn*L c–h) like individual 3. The diet of *O. cornuta* was then composed of: 4.3±0.3 families per nesting tube (mean number of families ± standard-error). The number of different plant families visited by *O. cornuta* detected *via trn*L g–h was 12, those *via trn*L c–h 12 and those *via* ITS 3 (average of 9.0 ± 3.0 families detected per marker). Furthermore, into these families identified in the nesting residuals by *O. cornuta*, the three most represented genera were *Acer*, *Quercus* and *Prunus*.

Only few families were shared between individuals, the highest proportions of shared families being between the different markers for a same individual (fig. 3). For instance, three families were found with both *trn*L g–h and *trn*L c–h in individual 3, and four families were found with both *trn*L g–h and *trn*L c–h in individual 2. Families shared between individuals

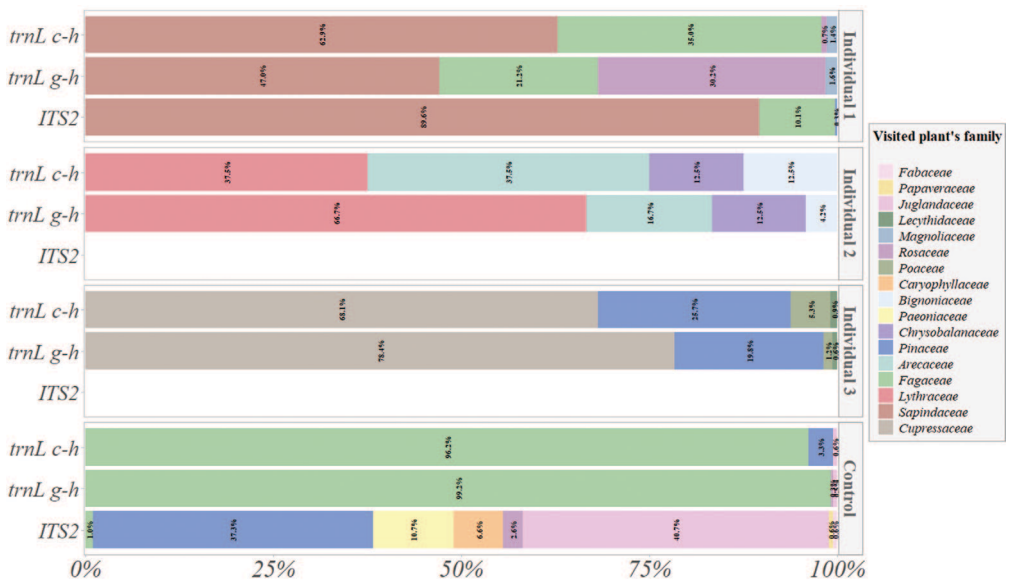

Fig. 2. – Bar plots of families of flowers visited by *Osmia cornuta* (Latreille) (individuals 1, 2, 3) and control *O. bicornis* (control individual) according to the marker used for the DNA detection (ITS2, *trn*L g–h or *trn*L c–h). DNA data filters: identifications that were found in at least two markers out of three for the same sample, and identifications from the family whose number of reads exceed 0.1% for a given sample and for a given marker. See Materials and Methods section for explanation about the zero detection of ITS2 in some of the samples.

were not numerous, but Pinaceae were found in both the control individual, individual 3 and individual 1, and Fagaceae and Rosaceae were found in both the control individual and individual 1.

***DNA markers comparison in the detection of plants visited by* Osmia cornuta *and visited plants in the control sample*.** – Variations in the number of taxa visited by bee individuals that have nested in the different sampled tubes were highlighted as follows (table IIa): 22 taxa for individual 1 (33.8% of all the 65 detected taxa), seven taxa for individual 2 (10.8% of all the detected taxa), eight taxa for individual 3 (12.3% of all the detected taxa) and 35 taxa for Finnish control individual *O. bicornis* (53.8% of all the detected taxa). The marker *trn*L g–h detected the highest number of overall taxa in the samples (33; 50.8% of the data), followed by ITS2 (28; 43.1% of the data) and *trn*L c–h (24; 36.9% of the data). The taxa were thus composed of 11 genera for individual 1 (four with ITS2, eight with *trn*L g–h and five with *trn*L c–h), three genera for individual 2 (one with *trn*L g–h and three with *trn*L c–h), four genera for individual 3 (all detected by both *trn*L g–h and *trn*L c–h) and 19 genera for control individual (11 with ITS2, eight with *trn*L g–h and five with *trn*L c–h). As some identifications went no further than the taxonomic resolution of families, it was possible that there were more families than genera for each individual. Data were represented by five families for individual 1 (three with ITS2, four with *trn*L g–h and four with *trn*L c–h), four families for individual 2 (all detected by both *trn*L g–h and *trn*L c–h), four families for individual 3 (all detected by both *trn*L g–h and *trn*L c–h) and eight families for control individual (eight with ITS2, three with *trn*L g–h and three with *trn*L c–h).

A total of 36 taxa went to the species taxonomic resolution. According to the number of plant species found in the samples (table IIb), there was 27.8% of the total number of species that were detected for individual 1 (ten species), 5.5% of total

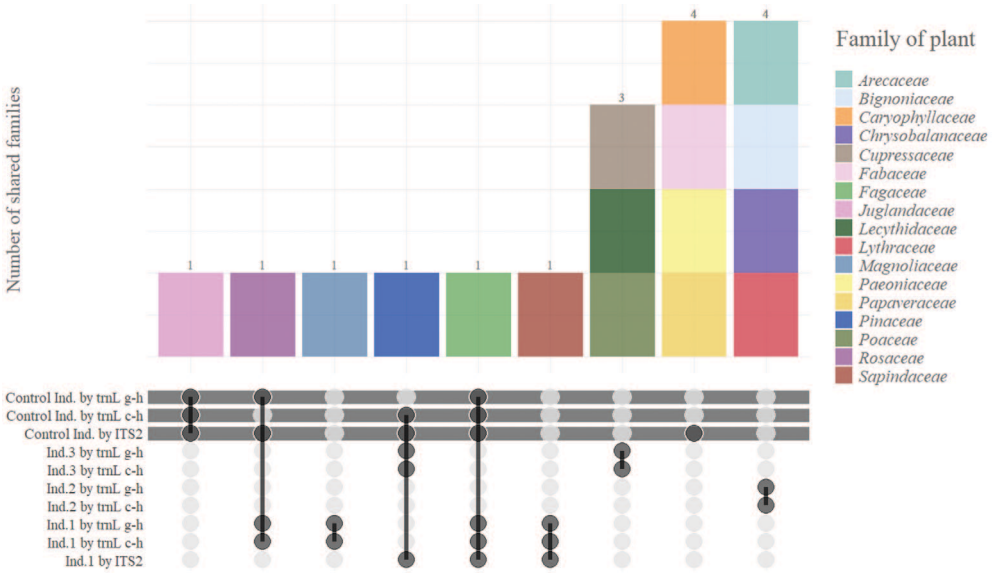

Fig. 3. – Number of shared species between the different individuals and markers. Black points represent the individual and the molecular marker considered in the calculation, whereas light grey points represent the individual and the marker that are excluded from the calculation. Number of shared families are represented by the histogram bars, above the individual and marker assemblage they represent.

number of species for individual 2 (two species), 5.5% of total number of species for individual 3 (two species) and 63.9% of total number of species for Finnish control individual (23 species). On the entire data set, the 36 detections down to species level varied between markers: 23 by ITS2 (63.9%), ten by *trn*L g–h (27.8%), and seven by *trn*L c–h (19.4%). ITS2 had a taxonomic resolution down to species level for nearly three-quarters of the taxa detected (71.4% for individual 1 and 86.4% for control individual; no detection for individuals 2 and 3), whereas *trn*L markers were less effective at going down to species level (individual 1: 35.7% for *trn*L g–h, 22.2% for *trn*L c–h; individual 2: 20.0% for *trn*L g–h, 40.0% for *trn*L c–h; individual 3: 20.0% for *trn*L g–h, 40.0% for *trn*L c–h; control individual: 30.0% for *trn*L g–h, 16.6% for *trn*L c–h).

Correspondence analysis (CA) showed a high segregation between families foraged by individual 2 and families foraged by individual 3, but individual 1 and control individual were poorly represented (fig. 4). Indeed, the further represented taxa from the origin and thus the most discriminated were those associated with *trn*L markers, which were strongly associated with a small number of families. This is why the first axis explained 33.7% of the variance in the data and segregated the individual 2, and the second axis explained 26.7% of the variance in the data and segregated the individual 3. For instance, families such as Lythraceae, Arecaceae or Bignoniaceae were strongly associated with the individual 2 with *trn*L markers.

Table II. – Table of samples by marker counting the number of taxa. – **a**, Taxa detected. – **b**, Taxa detected down to species level resolution.

		(a) **Number of taxa found**			
		ITS2	*trn*L g-h	*trn*L c-h	**Total of unique taxa for *O. cornuta* only**
Number of taxa found	Individual 1 (*O. cornuta*)	7	14	9	22
	Individual 2 (*O. cornuta*)	0	5	5	7
	Individual 3 (*O. cornuta*)	0	5	5	8
	Control individual (*O. bicornis*)	22	10	6	0
	Total of unique taxa for *O. cornuta* only	7	24	19	37

		(b) **Number of species found**			
		ITS2	*trn*L g-h	*trn*L c-h	**Total of unique species for *O. cornuta* only**
Number of species found	Individual 1 (*O. cornuta*)	5	5	2	10
	Individual 2 (*O. cornuta*)	0	1	2	2
	Individual 3 (*O. cornuta*)	0	1	2	2
	Control individual (*O. bicornis*)	19	3	1	0
	Total of unique species for *O. cornuta* only	5	7	6	15

Cupressaceae, Poaceae and Lecythidaceae seemed highly associated with each other, and part of the diet of individual 3. But for individual 1 and control individual, ITS2, *trn*L c–h and *trn*L g–h were close to the origin, as they were not differentiated based on any of the data in these samples.

DISCUSSION

The objective of this study was to non-lethally describe the plants visited by three individuals of European orchard bee *O. cornuta* by metabarcoding, through the use of three DNA markers on nesting residuals. Some results were expected, namely that markers detected approximately the same composition of flowers in the diet of one individual and that shorter markers would detect more taxa than longer markers. But we found that the individuals did not visit the same families as each other, even in the same geographic region —a bias most likely due to the very small number of samples in our study. The control individual, which was thought to have a very different diet from our three individuals, was as different from the French individuals as the French individuals were from each other.

According to the results, the diet of *O. cornuta* through metabarcoding analysis reflected its polylectic behaviour: it was composed of 37 taxa including a minimum of 12 different families, 19 different genera and 15 different species. Despite a little sampling effort, it was comparable to previous studies on *O. cornuta*: 12 different families and 33 different species in CASANELLES-ABELLA *et al.* (2022) by metabarcoding (ITS2 marker), 16 different taxa (genera or species level) in KRATSCHMER *et al.* (2020) by metabarcoding (*trn*L markers), eight families in HAIDER *et al.* (2014) by microscopy identification, 25 different taxa (genera or species level) in ECKERTER *et al.* (2022) by microscopy identification. We sampled both faeces and pollen as in FERNANDES *et al.* (2022) to maximize the detection of taxa, but it seemed to give same amount and resolution of taxa as studies on pollen balls only (KRATSCHMER *et al.*, 2020; CASANELLES-ABELLA *et*

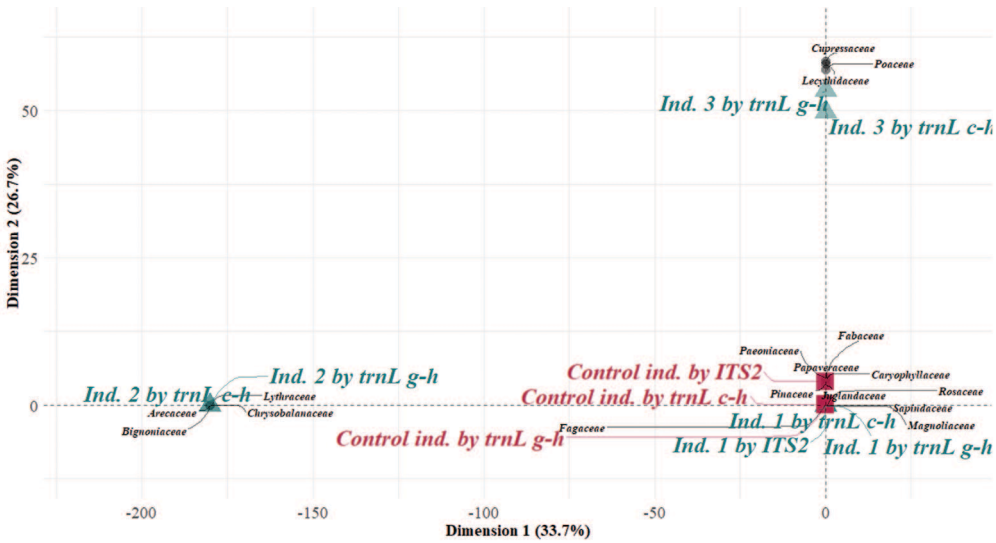

Fig. 4. – Biplot of the correspondence analysis at the plant family level for *Osmia cornuta* (Latreille) diet (individuals 1, 2, 3) and control *O. bicornis* diet (control individual). Red squares: control individual and the associated markers; blue triangles: individuals 1, 2, 3 and the associated markers.

al., 2022). Better understanding of the contribution of each cell component in determining the diet of mason bees is a challenge for future literature, pollen brood provisions being the most used component at present (Vaudo *et al.*, 2020 ; Crone *et al.*, 2025).

Casanelles-Abella *et al.* (2022) studied the families foraged by *O. cornuta* in France (Paris) and found Sapindaceae, Salicaceae and Rosaceae as the major plants detected by metabarcoding, which was consistent with our results: we found Sapindaceae, Fagaceae and Rosaceae as the three most visited families —regardless of the molecular marker (which are very classic urban trees; see Ossola *et al.* (2020)). Eckerter *et al.* (2022) found the following pollen genera as the most abundant in the diet of *O. cornuta*: *Prunus*, *Salix* and *Acer*. Kratschmer *et al.* (2020) found *Sorbus*, *Salix*, *Quercus* and *Acer* as the most represented pollen. In the present study, we found a slightly similar genera composition of *O. cornuta* diet with the highest number of reads —regardless of the marker— being: *Acer*, *Quercus* and *Prunus*. Haider *et al.* (2014) also found a tenth of *Salix* in *O. cornuta* diet through microscopic identification, that we did not find. The absence of Willow *Salix* in our data —Salicaceae family was removed by filters applied on DNA raw data to get more accurate results— can possibly be explained by the fact that the studies of Eckerter *et al.* (2022), Haider *et al.* (2014) and Kratschmer *et al.* (2020) were carried out respectively in Germany, Austria and Switzerland, three nearby regions, although the genus *Salix* is widespread throughout Europe (Wu *et al.*, 2015). It could be a question of method since two of the three papers previously cited used microscopy rather than metabarcoding. It also could be surprising that, before applying conservative filters on DNA results, we found *Ranunculus* in our raw data, and a diet composed of almost Ranunculaceae has a lethal effect on *O. cornuta* larvae (Eckhardt *et al.*, 2014) —even though it has been described as one of the family present in the diet of *O. cornuta* by metabarcoding (Casanelles-Abella *et al.*, 2022). But the presence of a diversified diet could be the result of a strategy of *O. cornuta*, with the aim of supplementing nutritional imbalances and attenuating the harmful secondary metabolites of some unfavourable pollen from Ranunculaceae (containing alkaloids, lactones, diterpenes or cyanogenic glycosides), as well as the aim to optimise the quality of the larvae's diet (Eckhardt *et al.*, 2014).

These studies on floral composition of mason bees' diets need completion by additional analyses of other aspects of the dietary niche, such as proteins or lipids contents. This range of approaches to describe the dietary niche was highlighted in particular by Crone *et al.* (2022), and then applied by Crone *et al.* (2023). The identity of the plants visited and their diversity is often the measure that is most emphasized and studied in the case of pollinators decline or resilience of pollination ecological networks (e.g. Kovács-Hostyánszki *et al.*, 2019 ; Kratschmer *et al.*, 2020 ; Splitt *et al.*, 2021), but the nutritional quality of the pollen collected by the bees is a crucial additional factor in determining the sustainability of these bees populations (Requier *et al.*, 2015; Cane, 2016; Kämper *et al.*, 2016).

Metabarcoding appears to be a very useful tool for determining the diet of mason bees, provided that the appropriate markers are chosen (Prosser & Hebert, 2017): on the one hand, the two *trn*L markers did not identify many plants beyond the family level, but on the other hand, the ITS2 marker detected only a few species. Combining several molecular markers therefore seems a good solution to overcome the drawbacks of each type of marker, but increases the cost of the analysis. Another limitation of using metabarcoding is the conservation of pollen. Indeed, in addition to not finding traces of pollen that have been eaten by the larvae, the glandular secretions of females

during nest construction tend to digest the cytoplasm of pollen grains and alter their structure (Ladurner *et al.*, 1999).

By exploring the floral diversity of the diet of *O. cornuta* through metabarcoding and using several DNA markers, the present study provides a basis to generalize this type of study on more ambitious programs with more complex experimental design and samples. The use of nest pollen and faeces residues has the advantages of being non-invasive, easy to replicate, and efficient to detect a wide variety of plant families, similar to those detected by microscopy analyses. As well as previous literature on artificial cavity-nesting bees from Megachilidae family, this study highlights the potential of applying non-lethal technique on *O. cornuta*. Even though it remains impossible to extend this to ground-nesting bees without destroying nests, this paper participates in more-in-depth knowledge on Megachilidae species diet description through metabarcoding using several molecular markers, following the ones on *Heriades sp.*, *Megachile sp.* or other *Osmia sp.*

Author contributions. – Conceptualization, CG, PC and BP; methodology, CG, PC, BP, JT and FM; formal analysis, CG; writing-original draft preparation, CG, PC, BP, JT and FM; writing-review and editing, CG, PC and BP. All authors have read and agreed to the published version of the manuscript.

Conflict of interest. – The authors declare that they have no conflict of interest.

Data availability. – Data will be made available on request to benjamin.poirot@apinov.com.

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